THE EFFECT OF AGE AND EXERCISE ON SERUM OSTEOCALCIN CONCENTRATION IN HORSES

By

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THE EFFECT OF AGE AND EXERCISE ON SERUM

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IN HORSES

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CHAPTER I

INTRODUCTION

Optimal growth of foals is advantageous for horses intended for show or sale as weanlings or yearlings. Horses achieve 80% of their mature body weight by 18 months of age (Evans et al., 1990). During this period of rapid growth and development, horses are weaned and begin physical conditioning, compounding concussion to long bones and joints (Young et al., 1991). It is a common practice in the horse industry to wean foals between four and six months of age (Evans et al., 1990). Age and rate of growth at weaning may have an effect on the rate of bone formation. Warren et al. (1997) reported reduced cannon circumference in foals weaned at 4.5 months of age compared to foals weaned at 6 months of age. These authors also reported that foals growing at a faster rate of gain at weaning encountered more growth depression.

Developmental orthopedic disease (DOD), a class of skeletal abnormalities that includes osteochondrosis, epiphysitis and flexural deformities, has become an increasing concern to producers. Explanation for the incidence of DOD is multifactorial (Pearce et al., 1998), but is evident that a method to identify the potential for developmental abnormalities is needed. Noninvasive markers of bone metabolism have

been used in humans for diagnosis and treatment of metabolic bone disease (Kruse and Kracht, 1986). Although biochemical markers of bone turnover are available (Hope et al., 1993), few have been used to monitor growth or skeletal disease in horses. Price et al. (1995) reported that horses with osteochondritis had altered serum osteocalcin concentrations.

Osteocalcin is a bone-specific protein synthesized by osteoblasts that is a specific marker of bone turnover in horses (Maenpaa et al., 1988). The effects of exercise on bone have been studied in rats (Westerlind et al., 1998), swine (Woo et al., 1981), poultry (Matsuda et al., 1986), and human subjects (Thorsen et al., 1997). There is also a potential to monitor skeletal response to exercise in horses using serum osteocalcin concentrations. A preliminary study by Julen Day et al. (1997) measured serum osteocalcin, Vitamin D, calcium and plasma IGF-I concentrations to monitor skeletal changes in two-year-old horses in race training. More research involving serum osteocalcin concentration in normal horses is needed before these markers can be effectively used interpret skeletal abnormalities.

CHAPTER II

LITERATURE REVIEW

Normal Bone Development

Woven bone and lamellar bone are the two main types of bone found in the skeleton. Woven bone is the immature form, composed of coarse random collagen fibers and lamellar bone is a highly organized and layered which is typical in the mature skeleton (Burkitt et al., 1993). During development, woven bone is produced initially, and eventually replaced by lamellar bone (Brighton et al., 1994). Mature bone consists of two distinct regions: a highly dense outer layer termed cortical bone and highly convoluted trabecular or cancellous bone that forms cavities occupied by the bone marrow. Bone is a dynamic tissue that is continually undergoing remodeling, the process of resorption of old bone and formation of new bone, to accommodate calcium homeostasis and changing mechanical stresses (Burkitt et al., 1993).

There are two major types of cells involved in bone metabolism: osteoblasts which are responsible for new bone formation, and osteoclasts which resorb bone (Christenson, 1997). Osteoblasts secrete and synthesize osteoid, the organic component of the extracellular matrix

of new bone (Brighton et al., 1994). Osteoblasts are then trapped within the bone as osteocytes and are responsible for maintenance of the bone matrix (Burkitt et al., 1993). Osteoclasts are multinucleate cells actively involved in the resorptive processes of the continual remodelling of bone (Burkitt et al., 1993). On the bone surface, metabolism by osteoclasts and osteoblasts occurs at specific sites referred to as bone metabolism units (BMU), which work in concert to maintain the intricate balance between formation and resorption of the entire skeleton (Christenson, 1997).

Postnatal foal growth of long bones, pelvis and vertebrae are characterized by endochondral ossification, a model of continuously growing cartilage replaced by bone (Burkitt et al., 1993). Bone growth occurs at the epiphseal plate (growth plate) which is the junction between the diaphysis (shaft) and the epiphysis (cartilagenous end) on either articulating end of the bone (Burkitt et al., 1993). At this junction chondrocytes (cartilage cells) mature, die and are replaced by bone, increasing the length of the diaphysis and pushing the growth plates further and further apart (Brighton et al., 1994). At maturity hormonal changes inhibit cartilage proliferation and growth plates are replaced by bone that effectively fuse the epiphysis and diaphysis (Burkitt et al., 1993). Horses attain 80% of their mature weight by 18 months of age, suggesting that the majority of long bone growth and weight gain happens during this period (Evans et al., 1990). Growth curves for wither height and animal weight support this conclusion (Figure 1; Figure 2).

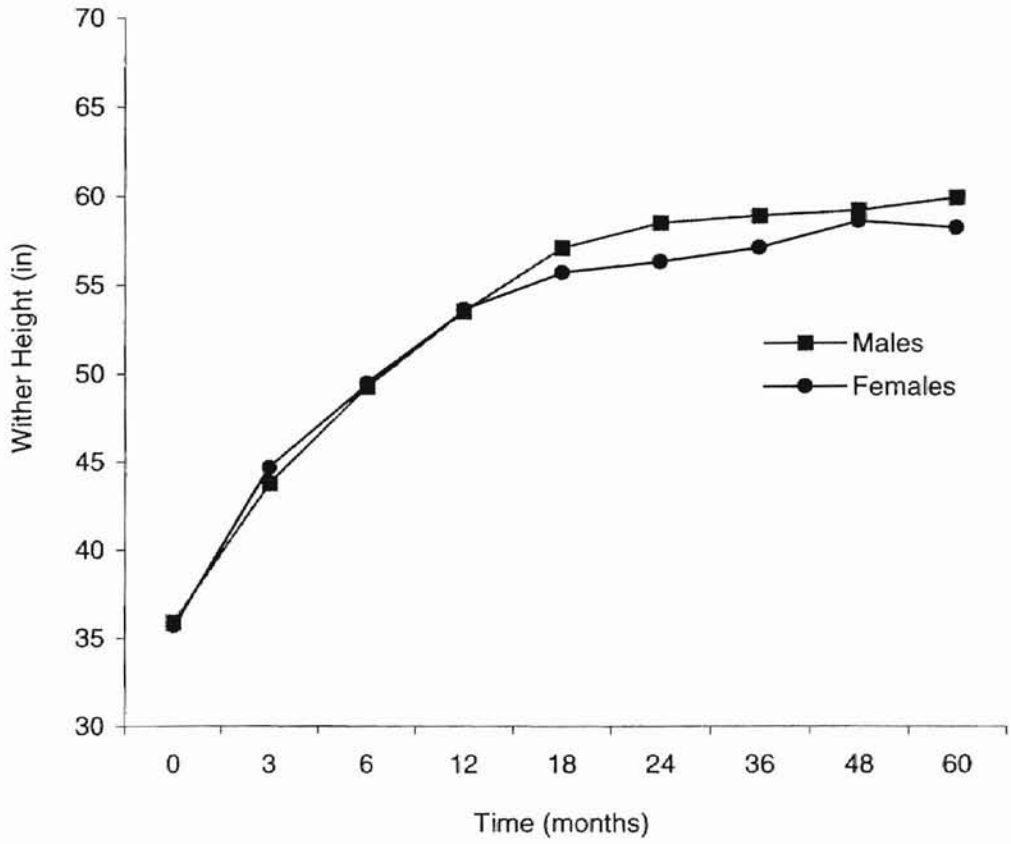

Figure 1. Wither Height of Male and Female Quarter Horses from Birth to 60 months of age (Cunningham and Fowler, 1961).

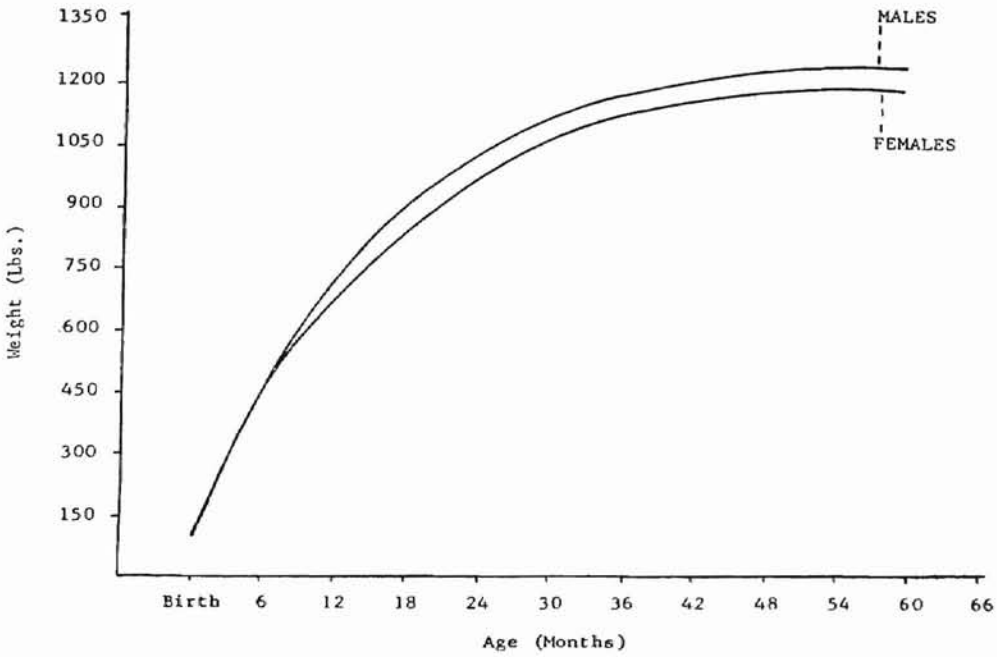

Figure 2. Growth Curve of Male and Female Quarter Horses from Birth to 60 months of age (Cunningham and Fowler, 1961)

Osteocalcin as a Biochemical Marker of Bone Metabolism

The rate at which new bone is formed and old bone is reabsorbed is termed bone turnover. Traditional methods of quantifying bone turnover rates utilize calcium kinetics and histomorphometric analysis of bone biopsies (Charles et al., 1985; Eriksen et al., 1993). Specific and more simplified noninvasive techniques for assessing bone metabolism in vivo are becoming more desirable and readily available (Christenson, 1997). Biochemical markers of bone turnover are equally useful in predicting bone formation and resorption during growth, as well as age-related changes due to osteoporotic weakening of the skeleton and metabolic bone disease (Weaver et al., 1996).

Currently validated markers specific for bone formation and turnover include the bone isoenzyme of alkaline phosphatase, propeptides derived from the Type I procollagen molecule, and osteocalcin also known as BGP or bone GLA protein (Russell, 1997). Alkaline phosphatase is not as desirable a marker of bone formation because its activity can be derived from sources other than bone (Kruse and Kracht, 1986). However, there is a significant inverse correlation between osteocalcin and alkaline phosphatase activity in horses (Lepage et al., 1990) and in patients with metastatic bone disease (Coleman et al., 1988).

Preliminary clinical cases report alterations in serum carboxyterminal propeptide of type I procallagen (PICP) and bone specific isoenzyme of alkaline phosphatase (BALP), in horses with osteochondritis

(Price et al., 1995). There is a potential for using serum osteocalcin concentrations to monitor bone metabolism in young horses undergoing rapid growth (Black et al., 1997) and training (Buckingham and Jeffcott, 1991; Julen Day et al., 1997). Osteocalcin has also been useful in monitoring bone mass and calcium accretion in humans during adolescence, which could help prevent age-related osteoporotic weakening of the skeleton (Weaver et al., 1996). In healthy females age 11-32 yr, a model utilizing osteocalcin and sexual maturity (postmenarcheal age) predicted 75% of the variability in calcium retention when consuming the recommended dietary requirements (Weaver et al., 1996). Osteocalcin concentrations may also provide an early indicator of successful therapy of bone disease since it is closely correlated to skeletal healing in osteopenic bone disease (Price et al., 1980). Elevated serum osteocalcin concentrations are also linked with vitamin D-dependent rickets, hyperparathyroidism, hyperthyroidism, bone metastases, Paget's disease, and renal osteodystrophy (Russell, 1997; Cole et al., 1985).

Osteocalcin Metabolism

Osteocalcin is a 5800 dalton protein that contains 3 γ-carboxyglutamic acid (Gla) residues and is the most abundant noncollagenous protein of bone (Price et al., 1976). Osteocalcin in the human, monkey, cow, rat and chicken have structural similarities including the sequence positions of its 3 γ-carboxyglutamic acid residues (Hauschka

et al., 1983). The Gla residues allow osteocalcin molecules to bind to Ca^{2+} ions and incorporate them into hydroxyapatite surfaces (Hauschka and Carr, 1982). Osteocalcin in synthesized by osteoblasts and requires the presence of vitamins D and K (Figure 3). Vitamin K is required for post-translational carboxylation of the glutamic acid residues during synthesis (Esmon et al., 1975). 1,25-dihydroxyvitamin D_3 (calcitriol) stimulates osteocalcin biosynthesis and secretion by rat and human osteoblasts (Price and Nishimoto, 1980; van Leeuwen et al., 1996; de Pollak et al.,1997), and is positively correlated with osteocalcin at the time of puberty (Ilich et al., 1997). The small fraction of newly synthesized protein that fails to bind to hydroxyapatite is released into the circulation. Osteocalcin is metabolized by the body with a half-life of 4-5 min and is cleared by the kidney (Price, et al., 1981). Impaired renal function in humans results in substantially increased concentrations of circulating osteocalcin due to diminished renal clearance of the protein (Cole et al., 1985).

Hormonal Regulation

Steroid hormones, growth factors, vitamins and cytokines play a role in the regulation of bone growth (Brighton et al., 1994). Sato et al. (1987) located receptors on osteoblastic cells for androgens, estradiol, progesterone, corticosteroids, and thyroxine.

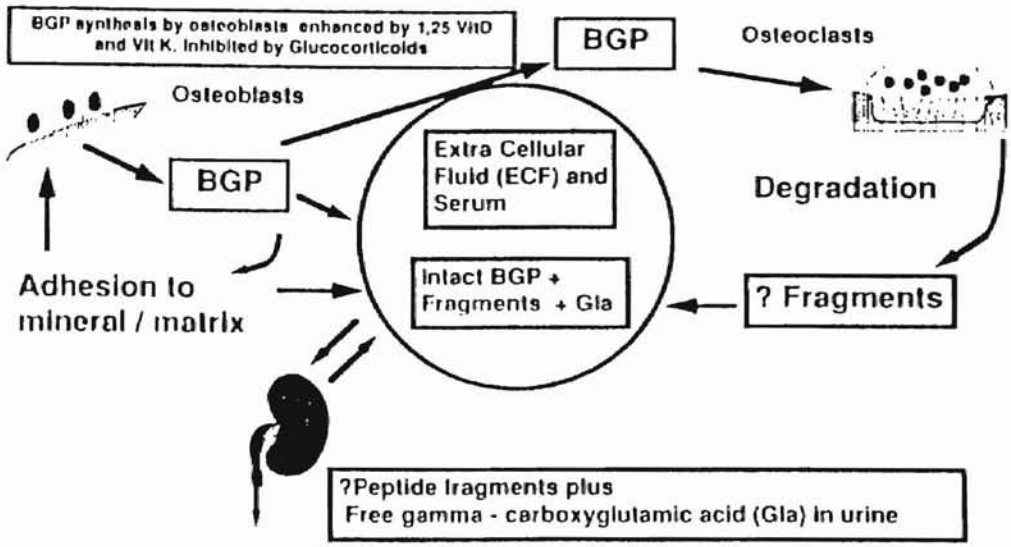

Figure 3. Proposed pathways for the biosynthesis and metabolism of osteocalcin. Synthesis of osteocalcin (BGP) by osteoblasts requires vitamin D, and vitamin K and is necessary for the post-translational modifications that give osteocalcin its binding properties (Russell, 1997).

Increased serum osteocalcin concentrations at puberty in boys due to increased serum testosterone concentrations have been suggested (Sorva et al., 1997). Increased serum osteocalcin concentrations in humans have also been associated with increased growth velocity and bone density following growth hormone therapy of growth hormone deficient children (Greig et al., 1997). Horses treated with equine somatotropin displayed elevated plasma IGF-I concentrations (Julen Day et al., 1997). Growth hormone affects skeletal growth indirectly by stimulating IGF-I release that binds to receptors on the target cells, including osteoblasts responsible for the production of osteocalcin (Brighton et al., 1994). Glucocorticoids reduce serum osteocalcin concentration by direct suppression of osteoblasts (Russell, 1997). Similarly, Patterson-Buckendahl et al. (1988) reported a negative correlation between corticosteroids and serum osteocalcin concentration.

In clinical studies involving calcium balance, no correlation between circulating osteocalcin concentrations and serum calcium concentration was evident (Weaver et al., 1996). Serum calcium was not correlated with osteocalcin concentration in normal men (Gundberg et al., 1985) or patients with metastatic bone disease (Coleman et al., 1988). However, parathyroid hormone and 1,25-$(OH)_2$ D_3, major regulators of calcium metabolism, play a role in regulating osteoblast function. Parathyroid hormone rapidly responds to a decrease in serum calcium, resulting in stimulation of renal 25-(OH)-D_3-1α-hydroxylase which eventually causes

an increase in serum 1,25-$(OH)_2$ D_3 in one proposed mechanism (Garabedian et al., 1972). Price et al. (1980) reported that 1,25 dihydroxyvitamin D_3 increases synthesis of osteocalcin. However, Brighton et al. (1994) suggest that vitamin D can exert opposing actions on the growth of bone depending on the maturational state of the target cells.

Circadian Rhythm

Some blood hormones and minerals associated with bone metabolism display circadian patterns. Serum phosphate, calcium (Markowitz et al., 1984), cortisol (Schlemmer et al., 1997), and parathyroid hormone (Jubiz et al., 1972) are reported to have a circadian rhythm. Bone formation itself is also diurnally regulated in rats with peak metabolic activity early in the photoperiod (Simmons and Nichols, 1966). Osteocalcin exhibits a circadian rhythm in humans (Beresford et al., 1985) and possibly in rats (Patterson-Buckendahl et al., 1988).

Limited research has been done concerning daily fluctuation in osteocalcin concentration in horses. Lepage et al. (1991) reported a biphasic circadian pattern in serum osteocalcin concentration of adult standardbred horses, characterized by relatively constant serum concentrations during the day followed by marked fluctuations during the dark hours. In contrast, no circadian pattern in equine serum osteocalcin was observed by Hope et al. (1993) in arabian horses. In this experiment,

fluctuations in serum osteocalcin occurred during the day but appeared random.

Variation in serum osteocalcin in horses has been attributed to transitions from light to dark and dark to light, corresponding to the light-associated alterations in circulating cortisol concentration (Lepage et al., 1992). Circadian variation in serum cortisol is a regulator of serum osteocalcin concentration in humans (Schlemmer et al., 1997). A similar circadian pattern in plasma cortisol and corticosterone has been elucidated in mares, characterized by a peak between 6:00 a.m. and 10:00 a.m. (Bottoms et al., 1972). In contrast, Hope et al. (1993) reported that osteocalcin concentration was independent of serum cortisol concentration. Variation in reported circadian rhythms of serum osteocalcin and cortisol in might be partially accounted for by the large age ranges of horses used in preliminary studies.

Effects of Age and Sex on Peripheral Osteocalcin Concentration

Equine Studies

Age is inversely correlated ($r=.75$, $P<.01$) with serum osteocalcin concentration in standardbred horses which is attributable to a significant reduction in the rate of bone formation and turnover of mature horses when compared with foals (Lepage et al., 1990; Lepage et al., 1992). Similarly, serum carboxyterminal propeptide of type I collagen (PICP), a biochemical marker of bone turnover was greater in thoroughbred foals

than in adult horses, reflecting differences in rate of bone remodeling (Price et al., 1995). Black et al. (1997) reported that plasma osteocalcin decreases from birth to one year of age in standardbred horses, and is highly correlated with foal body weight, girth, croup height and wither height.

Osteocalcin may also vary with breed and type of horse. Draft horses had significantly less serum osteocalcin when compared with warmblood horses of similar age (Lepage et al., 1997). Differences in hereditary morphologic and physiologic characteristics may explain the variation between draft horse and light horse breeds. Sex had no influence on serum osteocalcin concentration in standardbred horses under five years of age when age of the animal was included in the model (Lepage et al., 1997; Lepage et al., 1992).

Other Species

Osteocalcin has been studied in a variety of species, but the most extensive research has been done in the field of human medicine. Osteocalcin concentrations in infants of both sexes are similar at birth (Cole et al., 1985), but are greater in children than adults (Gundberg et al., 1983; Weaver, et al., 1996). Dramatic skeletal changes occur at the time of puberty and subsequent adolescence (Sorva et al., 1997). Additionally, Sorva et al. (1997) suggests a relationship between Tanner's stage of puberty and maximal bone matrix formation and mineralization. Cole et al. (1985) reported increases in serum osteocalcin in boys at 12

years of age and girls at 10 years, with peak concentrations achieved at 14 and 12 years of age, respectively. The peak of serum osteocalcin coincides with puberty, and is followed by a decline to adult levels between the ages of 18 to 20 years.

Serum osteocalcin concentrations in 450 normal children fluctuated with relation to age and sex, closely resembling normal growth (height velocity) curves for children (Johansen et al., 1988). Boys have higher peak osteocalcin concentrations at puberty than their female counterparts (Cole et al., 1985). Peak osteocalcin concentrations coincided with the pubertal growth spurt in boys (Sorva et al., 1997) and in girls (Johansen et al., 1988), at the same age as maximal height velocity was achieved. Post-menarchal girls have greater serum osteocalcin concentrations than their pre-menarchal counterparts, both groups strongly correlated with individual age and height (Weaver et al., 1996).

Cahoon et al. (1996) reported that male rhesus monkeys less than three years of age, had higher serum osteocalcin concentrations than their female counterparts. These researchers also detected a pronounced decline in serum osteocalcin concentration with age. Reported differences in osteocalcin concentration in relation to age and gender of the rhesus monkey are in agreement with human (Cole et al., 1985; Johansen et al. 1988) and equine studies (Lepage et al., 1990). Serum osteocalcin concentrations also decline with age in the rat, which parallel the decrease in bone turnover of the maturing skeleton (Patterson-

Buckendahl, 1988). Concentrations of osteocalcin vary with age both prenatally and postnatally, and can be used as an indicator of osteoblastic activity and bone formation in the ovine species (Collignon et al., 1996). Osteocalcin concentration decreased in fetuses prior to birth, and newborn lambs experienced a dramatic postnatal increase accompanied by a subsequent decline from 4 to 30 days of age. Osteocalcin is also an important predictor of bone mineralization in growing swine fed varying levels of calcium and phosphorous. Osteocalcin concentrations were inversely correlated with bone mineralization measurements and growth rate ($r=-.54$, $P<.01$) in growing pigs. (Carter et al. 1996). In summary, these data suggest that time and pubertal development, coupled with growth velocity are important variables in estimating serum osteocalcin concentration in addition to chronological age.

Effects of Exercise on Serum Osteocalcin Concentration

Equine Studies

Training is reported to elicit a mechanical and morphological response in thoroughbred horses, evidenced by an increase in bone density of the third carpal bone (Young et al., 1991). Similarly, two-year-old quarter horses in race training exhibited an initial decrease followed by subsequent increasing of medial and lateral cortical bone density during a 112 d training period (Julen Day et al., 1997). In addition, Porr et

al. (1997) reported increased bone mineral content in exercising horses fed diets high in calcium.

Osteocalcin may be a valuable noninvasive biochemical indicator of individual skeletal response to physical training in horses. The reported effects of exercise on serum osteocalcin concentration in horses is minimal in response to moderate exercise (Buckingham and Jeffcott, 1991).

Longitudinal Studies (>6 mo). Price et al. (1995) and Buckingham and Jeffcott (1991) reported differences in carboxyterminal propeptide of type I collagen (PICP), bone alkaline phosphatase (BALP) and plasma osteocalcin concentration reflecting small changes in bone turnover of exercised horses over a 12 month period. Exercising horses tended to have increased bone strength and density during exercise, and osteocalcin concentrations were significantly reduced when compared with sedentary animals (Buckingham and Jeffcott, 1991). Price et al., (1995) reported a decline in PICP and BALP, markers of bone formation, due to age-related changes during the sample period. Patterns of change between sedentary and exercised groups were different, characterized by a less pronounced decline initially in the exercised group, likely due to an early increase in bone turnover which attenuated the expected age-related decline in these markers (Price et al., 1995). Decline in serum osteocalcin concentration due to age can confound efforts to isolate the effects of exercise in longitudinal studies.

Short-term Studies (<6 mo). Julen Day et al. (1997) noted a decline in osteocalcin in exercised horses, which closely resembled changes in serum vitamin D concentrations. In the same study, osteocalcin concentrations of horses treated with exogenous equine somatotropin (eST) remained unaltered during this period. These authors postulated that horses treated with eST, undergo a period of greater bone turnover early in training. Osteocalcin concentrations were similar in both groups at the end of the 112 d treatment period, suggesting that horses experienced skeletal adaptation to exercise. Porr et al. (1997) also reported a linear decline in osteocalcin concentration over a 12 wk period in exercised horses. These studies support the idea that serum osteocalcin can be used to assess skeletal alterations due to exercise in horses.

Other Species

The effect of physical training on bone and its biochemical markers depends on the type, duration, and intensity of exercise combined with the age and nutritional status of the individual. Fitness level does not have an effect on osteocalcin concentration in adult men and women (Brahm et al., 1997). Incremental resistance (weight-bearing) exercise in adult rats resulted in an increase in osteocalcin mRNA expression in tibial bone when compared to control animals (Westerlind et al., 1998). In this study, control animals were subjected to the same exercise program as the treatment group without applied resistance (weight), indicating that the

type of exercise performed may dictate changes in bone turnover in response to exercise. A single bout of resistance exercise in young men failed to elicit the same response (Ashizawa et al., 1998). There is also evidence to suggest that very strenuous exercise may be detrimental to bone formation in young animals. Matsuda et al. (1986) reported that intense exercise in young roosters result in delayed long-bone growth. Exercise is thought to effect the quantity of bone rather than the quality of bone. Woo et al. (1981) concluded that mechanical and compositional properties of the bone remain unchanged following exercise, and accommodation of bone to exercise is a result of an increase in bone mass rather than composition. In support, Matsuda et al. (1986), and then Biewener and Bertram (1994) reported increased cortical thickness and cross-sectional area in response to exercise.

There are conflicting results concerning the effects of endurance exercise on serum osteocalcin concentration. Adolescent males experienced a 15% increase in serum osteocalcin concentration following 5 weeks of treadmill exercise (Eliakim et al., 1997). Single bouts of endurance exercise also elicit increases in serum osteocalcin and other markers of bone formation in humans. A single bout of exercise in young women caused an increase in bone turnover and altered calcium homeostasis, evidenced by changes in markers of bone formation, serum calcium and parathyroid hormone (Thorsen et al., 1997). Brahm et al. (1997) reported an immediate decrease followed by an increase in

osteocalcin concentrations of adult men and women 24 hr following a single intense exercise test.

In summary, it is evident that exercise does have an effect on bone, and more specifically serum osteocalcin concentrations. More research is needed to determine the nature of these changes with regard to age, sex and exercise, and to elucidate the mechanisms that regulate them.

Three studies were conducted to establish a normal range of serum osteocalcin concentrations in weanling and yearling Quarter horses with the following objectives: 1) to elucidate diurnal changes in osteocalcin concentrations in equine serum, 2) to determine if sex and/or weaning have an effect on serum osteocalcin concentrations of foals at four months of age, and 3) to evaluate the effects of sex and exercise on serum osteocalcin in yearling horses.

CHAPTER III

MATERIALS AND METHODS

Circadian Variation in Serum Osteocalcin in Quarter Horses

Eight weanling Quarter Horses were utilized to determine if serum osteocalcin concentration demonstrates circadian rhythm in horses at 4 and 11 months of age. This study is a combination of two trials: the first utilizing weanlings at 4 months of age (n=3) conducted in July (1997), and the second using weanlings at 11 months of age (n=5) in March (1998). All horses were fitted with 18 gauge indwelling jugular catheters secured with glue to the skin leaving the injection cap exposed. A 10% heparinized saline (5 mL) was used to flush the catheters after each blood sample was removed. To prevent damage to catheters, horses were tied with access to feed and fresh water. Horses were assimilated to the barn and lighting for two weeks prior to the study. Horses were exposed to 16 h daylight and 8 h of dark over the 24 h period, and feed was distributed twice daily at 7:00 a.m. (following blood sample collection) and 5:00 p.m. Blood samples were taken every 3 h for 24 h beginning at 7:00 a.m. Samples during the dark period were taken by flashlight to minimize exposure to light. Prior to sample collection 5 ml of blood was drawn

from each catheter and discarded to remove the heparinized saline. Blood was collected in 10 ml evacuated glass tubes (Vacutainer tubes, Becton, Dickinson & Co., Rutherford, New Jersey). Blood was allowed to clot at room temperature and sera were separated by centrifugation within 1 h and frozen at -20°C until analysis. Circulating osteocalcin can be measured in the horse with a radioimmunoassay (RIA) reported to have crossreactivity with human osteocalcin from bone and serum (Patterson-Allen et al., 1982), and validated for use in horses (Hope et al., 1993). Serum was assayed in 50 μl aliquots in duplicate using a commercially-available radioimmunoassay (Osteocalcin Radioimmunoassay Kit, Cat. #15130, Incstar Corporation, Stillwater, Minnesota) according to manufacturer's specifications with the following modification. Some samples contained higher concentrations of osteocalcin than the osteocalcin standard provided with the radioimmunoassay kit and therefore, did not fall on the standard curve. Dilutional parallelism for this radioimmunoassay was documented by Hope et al. (1993). Samples were reassayed using 25μl of sample with 25μl of 0 standard buffer to attain sample uniformity. The mean result was multiplied by 2 to correct for dilution. Interassay coefficient of variation was less than 9% and intraassay coefficient of variation was less than 4%. Mixed procedure of SAS (1996) for repeated measures was used to determine the influence of sampling time on serum osteocalcin concentration.

Effects of Sex and Weaning on Serum Osteocalcin Concentration in Horses

Twelve Quarter Horse foals were weaned at four months of age to evaluate changes in peripheral osteocalcin at weaning. Foals were removed from pasture and placed in catch pens (10 x 20 ft) at weaning. Two types of weaning were implemented; abrupt (Ab) and gradual (Gr). Abruptly weaned foals were completely separated from mares and removed from the premises. Gradual weaning allowed mare and foal to remain in close visual proximity, but prevented suckling for 2 d prior to removal of the mare. Horses were weaned in pairs to minimize stress. Sex of foal was recorded. Blood serum samples from the jugular vein were collected 1 d preweaning, immediately prior to weaning, 4 h postweaning, and daily for 1 wk postweaning. Blood samples were collected at 1:30 p.m. into 10 ml evacuated glass tubes (Vacutainer tubes, Becton, Dickinson & Co., Rutherford, New Jersey) and allowed to clot at room temperature. Samples were separated by centrifugation 1-2 hr following venepuncture and frozen for analysis. Osteocalcin in serum was quantified in duplicate using a commercially available radioimmunoassay kit (Osteocalcin Radioimmunoassay Kit, Cat. #15130, Incstar Corporation, Stillwater, Minnesota) for human serum, validated for use in horses (Hope et al., 1993). The radioimmunoassay kit utilizes bovine osteocalcin standard reported to crossreact with equine osteocalcin (Patterson-Allen et al., 1982), and was used with the following modification. A few samples contained higher concentrations of

osteocalcin than the osteocalcin standard provided with the radioimmunoassay kit, which did not fall between standard curve values. Samples were reassayed using 25μl of sample with 25μl of 0 standard buffer to attain the required volume of 50μl. The mean result was multiplied by 2 to correct for dilution. Interassay coefficient of variation was less than 8%. Split-plot analysis of variance was used to determine the influence of sex (Table 1) and weaning method (Table 2) on serum osteocalcin. Means were compared by protected least significant differences (SAS, 1996).

The Effect of Age and Exercise on Serum Osteocalcin Concentration in Yearling Horses

Fifteen Quarter Horse yearlings (7 geldings, 8 fillies) were utilized to quantify serum osteocalcin in sedentary and exercising horses. Fifteen non-exercising yearling horses were placed in a 40 acre pasture and blood serum samples were collected 0, 45, and 90 d following placement in pasture. After day 90, the same horses were subjected to light exercise for approximately 1 h three times per week for 12 weeks. Jugular blood samples were taken 45 and 90 d following initiation of the exercise program. All blood samples were collected in 10 ml evacuated tubes (Vacutainer tubes, Becton, Dickinson & Co., Rutherford, New Jersey) at 1:30 p.m. the appropriate day. Sera were separated by centrifugation within 1 h following collection and frozen until analysis. Serum osteocalcin was quantified in duplicate by radioimmunoassay (Osteocalcin

Radioimmunoassay Kit, Cat. #15130, Incstar Corporation, Stillwater, Minnesota) according to manufacturer's specifications. Interassay coefficient of variation was less than 2%. Intraassay coefficient of variation was less than 4%. Separate analysis of variance using a split-plot design was used to evaluate serum osteocalcin concentration in sedentary (Table 3) and exercising horses (Table 4). Means were compared by protected least significant differences (SAS, 1996).

Table 1. Analysis of variance table illustrating the effects of sex and horse on sample time and appropriate interactions at weaning.

Source	Degrees of Freedom
Sex	1
Horse(Sex)	10
Time	9
Time x Sex	9
Residual	88

Table 2. Analysis of variance table for the effects of horse and weaning method with appropriate interactions on osteocalcin concentration.

Source	Degrees of Freedom
Wean	1
Horse(Wean)	10
Time	9
Time x Wean	9
Residual	88

Table 3. Analysis of variance table illlustrating the effects of horse and sex with appropriate interactions on sample time in sedentary horses.

Source	Degrees of Freedom
Sex	1
Horse(Sex)	13
Time	2
Time x Sex	2
Residual	26

Table 4. Analysis of variance table for the effects of horse and sex with appropriate interactions on sample time in exercising horses.

Source	Degrees of Freedom
Sex	1
Horse(Sex)	13
Time	2
Time x Sex	2
Residual	26

CHAPER IV

RESULTS AND DISCUSSION

Circadian Variation in Serum Osteocalcin of Quarter Horses

Time of sampling did not influence ($P<.09$) serum osteocalcin concentration during the 24 hr sample period in foals at either 4 or 11 months of age (Figure 2). In support, Hope et al. (1993) did not observe a pattern in serum osteocalcin concentration of arabian horses. Although there were small fluctuations (not significant) during the 24 hr sample period, no pattern was evident in these horses. Lepage et al. (1992) observed a biphasic circadian pattern in serum osteocalcin concentrations of adult standardbred horses. These authors postulated that changes in osteocalcin are stimulated by the transition from light to dark and dark to light, and might parallel fluctuations in serum cortisol concentration. Administration of corticosteroids results in a decline in serum osteocalcin concentration (Patterson-Buckendahl et al., 1988; Geor et al., 1995), and serum cortisol fluctuates diurnally in mares (Bottoms et al., 1972). However, serum cortisol is not correlated with osteocalcin concentration in horses (Hope et al., 1993). These authors suggested that continuous light during portions of the experiment may have interrupted fluctuations due to

light and dark cycles. In the present study, horses were exposed to both light and dark, and samples were taken by flashlight to minimize exposure to light during the dark period. Previous studies have mainly used mature animals as their experimental model. Osteocalcin concentrations are known to be affected by age (Lepage et al., 1990) and growth rate (Carter et al., 1996) of the animal. Differences in pattern of secretion of serum osteocalcin between young and old animals may exist. There may be a possible trend towards a diurnal pattern in the horses at 11 months of age.

Osteocalcin concentrations were affected ($P<.001$) by age of horse. Weanlings at 11 months of age had consistently greater osteocalcin concentrations than those at 4 months of age. Greater serum osteocalcin concentrations in the 11 month age group might be due to an increase in serum osteocalcin concentrations prior to puberty. Puberty occurs in horses between 12 and 15 months of age (Evans et al., 1990). This is in accordance with (Cole et al., 1985) who indicated, that serum osteocalcin concentrations increase prior to puberty in humans.

Effects of Sex and Weaning on Serum Osteocalcin Concentration in Horses

Serum concentrations of osteocalcin were similar to those of clinically normal horses studied by Hope et al. (1993). Serum osteocalcin concentrations were affected by sex of foal ($P<.05$). Colts had higher circulating osteocalcin concentrations ($19.3 \pm .6$ ng/mL) when compared with fillies ($17.2 \pm .5$ ng/mL) (Table 5). Johansen et al. (1988) found sex-

Table 5. Influence of sex and weaning method on serum osteocalcin concentrations of foals.

	Sex of Foal		Weaning Method	
	Colts	Fillies	Abrupt	Gradual
Osteocalcin, ng/mL	19.3 ± .6[a]	17.2 ± .5[b]	18.5 ± .7	17.7 ± .7

[a,b] Means within a group with different superscripts differ (P<.05).

related differences in serum osteocalcin concentrations of children. Differences related to sex may reflect differences in growth rates of colts and fillies. Male foals less than four months of age experience a greater rate of growth at limb bone extremities than their female counterparts (Goyal et al., 1981). Serum osteocalcin is affected by rate of growth in swine (Carter et al., 1996). Serum osteocalcin concentrations were not affected by sex in standardbred horses less than 5 years of age (Lepage et al., 1992). However, reproductive status and exercise regimes of horses used were variable. Sex-related differences in the present study might also be due to the influence of steroid hormones. Receptors for thyroxine, androgens, estradiol, progesterone and corticosteroids have been located on osteoblastic cells (Sato et al., 1987). However, the specific interactions between these factors and osteoblastic activity are largely unclear. Serum osteocalcin in all horses declined ($P<.05$) 1 to 2 d following weaning (Figure 3). This is in agreement with Maenpaa et al. (1988) that osteocalcin decreased when foals were transferred from pasture to stables. Osteocalcin concentrations were restored to preweaning levels within 7 d postweaning. Serum osteocalcin reflects bone formation, and therefore osteoblastic activity (Kruse and Kracht, 1986; Brown et al., 1984; Price et al. 1980). Warren et al. (1997) reported that weaning depressed the growth of cannon circumference in foals weaned at 4 months of age. In the same study, foals growing at a faster rate of gain encountered more growth depression at weaning. The

variation in osteocalcin at weaning might be due to acute stress associated with the weaning process. Movement of rats from group to individual housing resulted in a marked decline in serum osteocalcin concentrations after 24 hours due to corticosteroid activity (Patterson-Buckendahl, 1988). These animals also experienced a 17% decrease in osteocalcin following simple restraint. Serum osteocalcin concentrations are decreased by glucocorticoids in horses (Geor et al., 1995). In the present study, we might expect increased corticosteroids to influence serum osteocalcin concentration because foals were moved from pasture to pens at weaning, creating a more physically restrictive environment as well as increasing stress to the animal due to weaning.

Method of weaning did not affect ($P>.1$) changes in serum osteocalcin at weaning. Osteocalcin concentrations were 18.5 ± .7 ng/mL and 17.7 ± .7 ng/mL for abrupt and gradual methods, respectively (Table 5).

Effects of Sex and Exercise on Serum Osteocalcin Concentration in Yearling Horses

Serum osteocalcin concentrations of sedentary yearling horses were not affected by sex ($P<.1$). Colts and fillies had concentrations of 15.3 ± .7 ng/mL and 16.1 ± .6 ng/mL, respectively (Table 6). Lepage et al. (1992) concluded that serum osteocalcin concentrations of standardbred horses less than 5 year of age were not affected by sex. In contrast, osteocalcin concentrations were affected by sex in exercising horses

($P<.05$). Fillies had higher circulating osteocalcin concentrations (14.65 ± .5 ng/mL) when compared with colts (12.88 ± .6 ng/mL) (Table 6). Exercising measurements (September-December) were taken following the 90 d sedentary period (June-August). Due to this complication, horses were older during the exercise phase of the experiment. Girls tend to reach puberty and corresponding peak osteocalcin concentrations at an earlier age than boys (Cole et al., 1985). Fillies also tend to reach puberty earlier than colts (Evans et al., 1990), which might explain why fillies had higher osteocalcin concentrations in the present study.

Sedentary horses had similar ($P<.33$) osteocalcin concentrations at 0, 45, and 90 d (Table 7). Serum osteocalcin was lower ($P<.0006$) in all horses 45 and 90 d after initiation of the exercise program (Table 7). Osteocalcin decreased linearly ($P<.0001$) during physical conditioning (Figure 4). In agreement, Julen Day et al. (1997) and Porr et al. (1997) reported reduced serum osteocalcin concentrations of exercising horses.

. It is impossible to determine if the decline in osteocalcin is due to exercise or normal age-related changes in the present study. No age-matched control values were available to make comparisons regarding the nature of the decrease in osteocalcin. However, it is likely that the linear decrease in exercised horses in the present study, reflects the natural decline in osteocalcin concentration as animals reach skeletal maturity. Osteocalcin concentration decreases to adulthood in monkeys (Cahoon et al., 1996), rats (Patterson-Buckendahl et al., 1988), humans (Gundberg et

al., 1983) and horses (Lepage et al., 1990). However, no age-related decline was seen over the same period of time in the sedentary horses.

Osteocalcin concentrations are a reflection of bone formation and (or) turnover (Price et al., 1981; Kruse and Kracht, 1986). Generally, exercise increases bone turnover and serum osteocalcin concentration which differs from the results in our study (Eliakim et al., 1997; Thorsen et al., 1997; Brahm et al., 1997). Detrimental effects of exercise on bone are associated with very strenuous exercise (Matsuda et al., 1986). The light duration and intensity of exercise performed by horses in this study makes the possibility of detrimental skeletal affects unlikely. Price et al. (1995) concluded that exercise had a dampening effect on the natural decline of biochemical markers of bone formation. Rate of growth influences serum osteocalcin concentrations in swine (Carter et al., 1996). Horses were older during the exercise phase, so differences in serum osteocalcin concentrations of exercising horses might reflect alterations in growth rate and skeletal maturation at this age.

Table 6. Influence of sex on serum osteocalcin concentrations of sedentary and exercising yearling horses.

	Sedentary		Exercising	
Sex	Colts	Fillies	Colts	Fillies
Horse, no	7	8	7	8
Osteocalcin, ng/mL	15.31 ± .7	16.10 ± .6	12.88 ± .6[a]	14.65 ± .5[b]

[a,b] Means within a group with different superscripts differ (P<.05).

Table 7. Serum osteocalcin concentrations of sedentary and exercising yearling horses.

	Sedentary			Exercising	
Day	0	45	90	135	180
Osteocalcin, ng/mL	16.56 ± .7[a]	15.13 ± .7[a]	15.43 ± .7[a]	13.94 ± .5[b]	11.92 ± .5[c]

[a,b,c] Means within a group with different superscripts differ (P<.05).

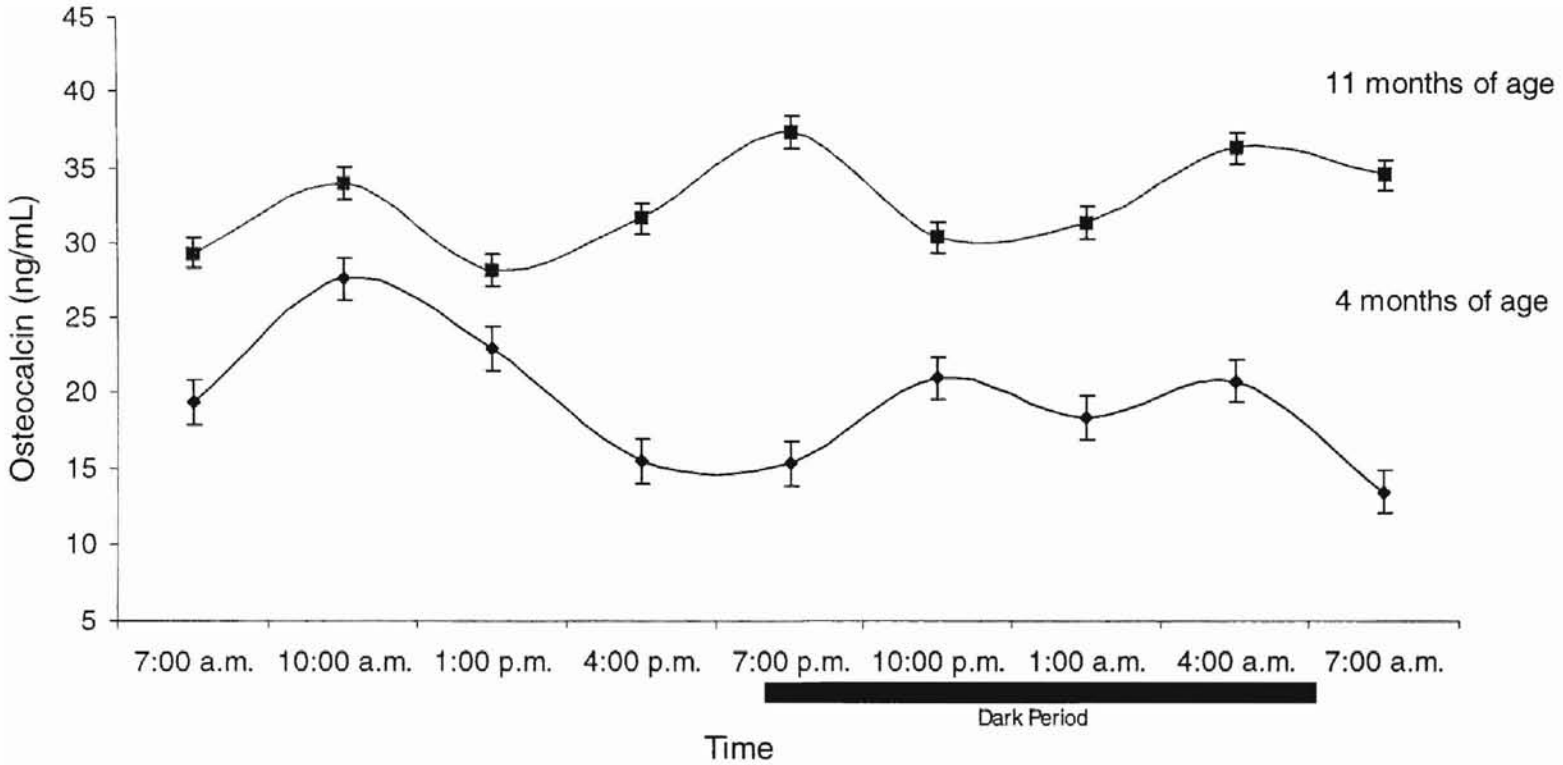

Figure 4. Serum Osteocalcin Concentrations of Horses During a 24 hr Period. There was no effect (P<.1) of sample time on serum osteocalcin concentration in weanlings at 4 and 11 months of age.

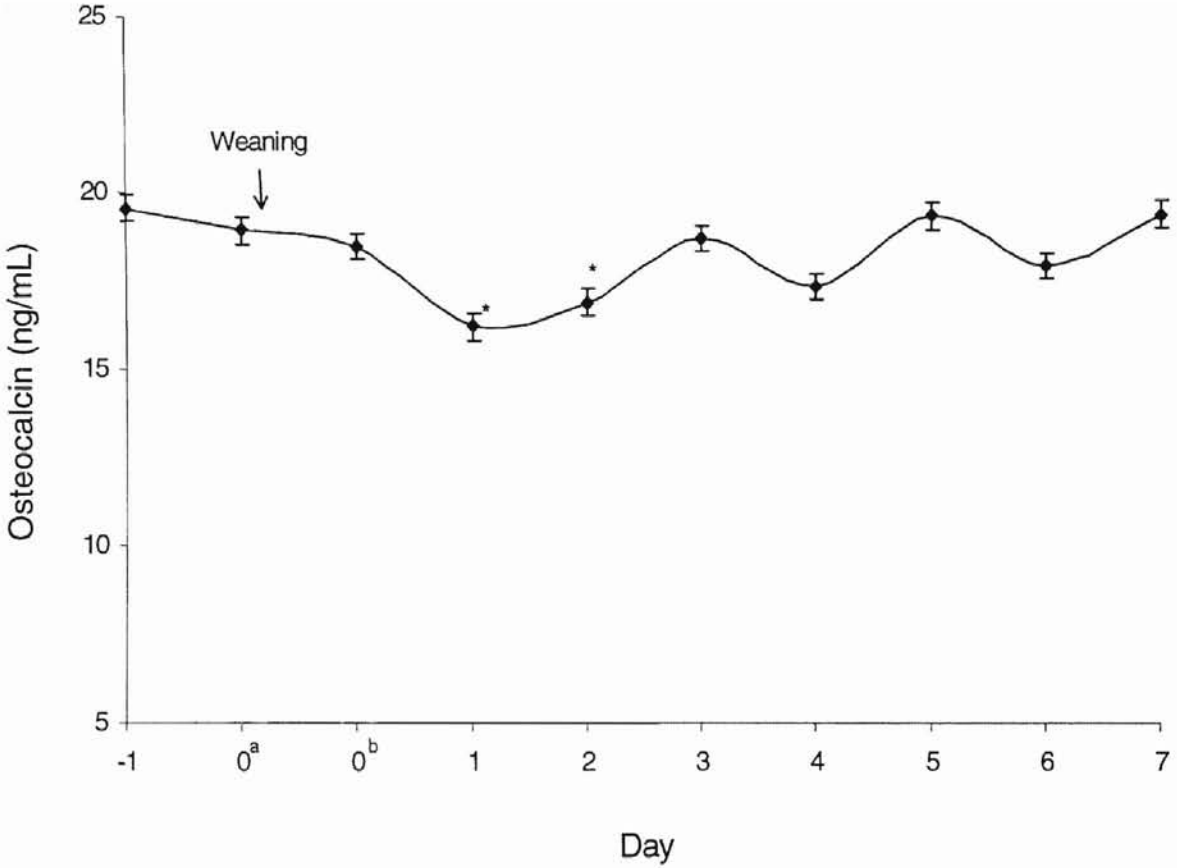

Figure 5. Serum Osteocalcin Concentrations at Weaning.
0^a denotes sample taken prior to weaning and 0^b denotes 4 h postweaning.
*Means are significantly different (P<.05).

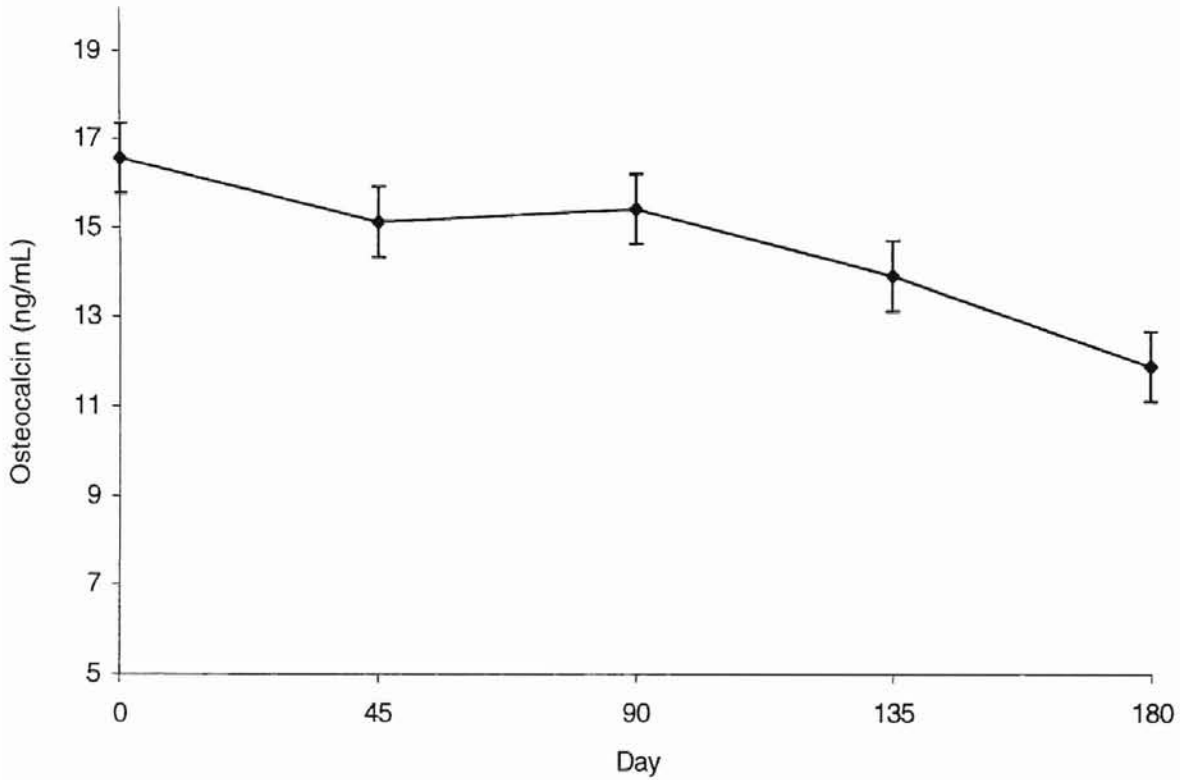

Figure 6. Serum Osteocalcin Concentrations of Sedentary and Exercising Yearling Horses. Serum osteocalcin concentrations of sedentary yearling horses were similar (P>.1) on days 0, 45, and 90. Exercising horses (days 135, 180) showed a linear decline (P<..0001) in osteocalcin.

CHAPER V

SUMMARY AND CONCLUSIONS

Three experiments were conducted to establish a normal range of serum osteocalcin concentrations in weanling and yearling Quarter Horses. The specific objectives of this research were: 1) to assess daily changes in osteocalcin concentration in equine serum, 2) to determine if sex and weaning affect serum osteocalcin in foals at four months of age, and 3) to evaluate the effects of sex and physical conditioning of serum osteocalcin in yearling horses. Eight weanling Quarter Horses at 4 months of age (n=3) and eleven months of age (n=5) were used in experiment 1 which was conducted in two parts. Horses were exposed to normal light and dark cycles with jugular blood samples taken at 3 hour intervals for 24 hours. Concentrations of osteocalcin were higher in the horses at 11 months of age, but were not influenced by time of sampling ($P<.09$) in either group of horses. More blood samples at shorter intervals (30 min or 1 hr) may be required to distinguish a pattern of osteocalcin secretion, if it exists. Lack of diurnal fluctuation suggests that regulating time of blood sampling for osteocalcin determinations in metabolic studies may not be necessary.

90, and performed light exercise three times per week. Sex did not influence P<.41) osteocalcin concentrations during the sedentary phase, but fillies had higher osteocalcin concentrations than colts (P<.04) during the exercise phase (14.65 ± .5 vs 12.88 ± .6 ng/mL, respectively). Age and pubertal development may have been responsible for this difference. Fillies tend to undergo puberty sooner than colts, and therefore may reach peak osteocalcin concentrations sooner than their male counterparts. Osteocalcin concentrations of all horses were similar (P<.33) during the sedentary phase. During exercise, osteocalcin concentrations decreased linearly (P<.0001). This decline in bone turnover during exercise could be related to the introduction of training, however it is more likely that it reflects the normal age-related decline expected as animals reach skeletal maturity. Lack of age-matched controls make conclusions about the effects of age and exercise on osteocalcin impossible.

In conclusion, osteocalcin concentrations of horses in all three studies were similar to those of clinically normal horses (Hope et al., 1993; Lepage et al., 1990). Sex-related differences in osteocalcin seem to be linked with age and development. Results of the three studies support the conclusion that osteocalcin can be used to assess bone metabolism during growth and training in young horses. Serum osteocalcin concentrations compared to normal values for the age and sex of the individual could be useful for breeders and trainers to evaluate expected growth and response to training.

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